Separation of endo- and exo-type cellulases using a new affinity chromatography method

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A new efficient biospecific adsorbent for the purification of cellulases was prepared from *p*-aminobenzyl 1-thio-β-D-cellobioside and Affigel-10 (Biorad). The two exocellobiohydrolases, CBH I and CBH II, from *Trichoderma reesei* QM 9414 were adsorbed, whereas the endoglucanase activities were unretarded. The adsorbed CBH I can be displaced selectively by elution with lactose, whereas cellobiose elutes the CBH II.

Cellulase *Exo-cellobiohydrolase* Trichoderma reesei *Endocellulase* *Affinity chromatography* *Enzyme purification*

1. INTRODUCTION

Cellulolytic microorganisms produce complex enzyme systems which present considerable fractionation problems. Ion-exchange chromatographic methods have been used most often [1,2]. Affinity chromatography on microcrystalline cellulose (Avicel) or on amorphous cellulose has been attempted by several authors [3–7]. The difficulties encountered in eluting adsorbed enzymes from these supports were discussed fully in [8].

We report a simple method for the purification of exo-type cellulases from *Trichoderma reesei* QM 9414 using a synthetic low-M_r ligand coupled to an inert support. Specific elution allows group separation of functionally related enzymes which are known [2] to appear as isoenzymes in the culture filtrates.

Abbreviations: CBH, 1,4-β-glucan cellobiohydrolase (EC 3.2.1.91); endocellulase, 1,4-β-glucan glucanohydrolase (EC 3.2.1.4); HPLC, high-performance liquid chromatography

2. MATERIALS AND METHODS

2.1. Preparation and use of affinity column

The *p*-nitrobenzyl 1-thio-β-D-glucopyranosides from cellobiose and lactose were prepared as in [9]: mp 246°C; $[\alpha]_D^{20}$ − 28° (*c* 0.5, pyridine) for the cellobioside and mp 238°C; $[\alpha]_D^{20}$ − 91° (*c* 0.5, pyridine) for the lactoside. The corresponding amine derivatives (0.5 g) obtained by catalytic reduction of the nitro compound [9] were coupled directly to Affigel-10 (Biorad) (25 ml) without further purification. For this purpose the gel, after washing with cold water (50 ml), was added to the amine derivative dissolved in 20 ml of 0.1 M sodium bicarbonate and the slurry left for 24 h at room temperature.

The resulting affinity carriers were loaded into cooled (4°C) glass columns (1 × 10 cm) and washed extensively with sodium acetate buffer (0.1 M, pH 5.0, 0.01% NaN_3). Chromatography was performed at 30 ml/h and the eluates monitored at 254 nm (Isco Model UA-4 absorbance unit).

2.2. Enzymes and enzymatic assay

Partially purified samples of exocellobiohydrolases I and II and endocellulase (from *T.reesei*

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QM 9414) were obtained by ion-exchange chromatography [1,2]. Lyophilized culture filtrates were used as starting material. 4-Methylumbelliferyl glycosides derived from lactose and cellooligosacchrides were prepared as in [10,11] and used as chromatophoric substrates for these enzymes. Spectrophotometric and HPLC methods were used to detect and measure the concentration of the reaction products [10].

Carboxymethylcellulase and Avicelase activities were measured by standard procedures using a colorimetric method (dinitrosalicylic acid reagent) to estimate the amount of reducing sugars liberated.

Protein concentrations were estimated from reported absorption coefficients at 280 nm [4] or by the method of Lowry using bovine serum albumin as standard.

Analytical isoelectric focusing was performed on an LKB Multiphore apparatus and Ampholine PAG plates (pH 3.5–9.5). Samples were first dialysed against distilled water using an Amicon dialysis apparatus (PM-10 filter).

3. RESULTS AND DISCUSSION

1-Thio-β-D-glycosides derived from cellobiose and lactose are powerful competitive inhibitors of CBH I from *T.reesei*. At neither the holosidic nor heterosidic bonds was significant hydrolysis of these compounds observed (HPLC analysis). As measured with the chromogenic 4-methylumbelliferyl 1-*O*-β-D-lactoside as substrate [10] the values for the inhibition constants were estimated: 2 mM for the *p*-nitrobenzyl 1-thio-β-D-lactoside and 0.33 mM for the corresponding cellobiose derivative (25°C, pH 5.0). We coupled the corresponding amino derivatives covalently to an affinity chromatography carrier (Affigel-10, Biorad).

CBH I (20 mg, purified by ion-exchange chromatography) from *T.reesei* was completely retained by the affinity support carrying the thiocellobioside as ligand, whereas no binding to the thiolactoside column was observed. Desorption was achieved by applying either 0.01 M cellobiose or 0.1 M lactose solutions (pH 5.0), whereas attempts to elute with KCl (1 M), ethylene glycol (1 M) or glucose (1 M) solutions were unsuccessful. The specificity properties of the enzyme purified by affinity chromatography coincide with those reported [10].

Partially purified (ion-exchange chromatography) CBH II samples (5–20 mg) were similarly applied to the thiocellobioside column. Some protein eluted with buffer, whereas only cellobiose (0.01 M) could desorb the bound fraction. The specificities of the unretarded and adsorbed fractions differed considerably when the activities toward the 4-methylumbelliferyl derivatives of the lower cellooligosaccharides were investigated (not shown). The activity profile of the adsorbed enzyme is typical for an exocellobiohydrolase type cellulase. The purity of the adsorbed fraction was demonstrated by isoelectric focusing (fig.2). The specificity of the non-adsorbed fraction is that of an endo-type cellulase [10].

The affinity support carrying the cellobiosyl moiety is an effective adsorbent of exo-type cellulases, whereas the lactoside column fails to adsorb these enzymes. Although at least CBH I shows affinity for lactose and its derivatives [10], attachment of these ligands to an affinity support seems to reduce the binding capacity considerably.

Clearly, a biospecific adsorbent for CBH I and CBH II has been prepared and the selective desorption should permit successive elution of both enzymes from a complex mixture. Fig.1 illustrates the purification procedure used for a crude cellulase preparation. D-Glucose (1 M) or gluconolactone (1 mM) were added to all equilibrating and eluting buffer solutions. The presence of these

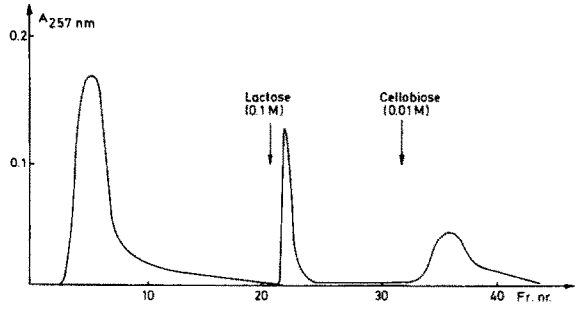

Fig.1. Affinity chromatography of crude cellulase from *T.reesei*. Crude cellulase (lyophilized, 50 mg), dissolved in 5 ml of 0.05 M sodium acetate buffer, pH 5.0 (0.01% NaN_3) containing 1 mM gluconolactone, was applied to the affinity carrier packed in a column (1 × 10 cm) (4°C), equilibrated with the same buffer solution. Elution (30 ml/h) was continued first with the same buffer, then with buffer supplemented with 0.1 M lactose or 0.01 M cellobiose as indicated.

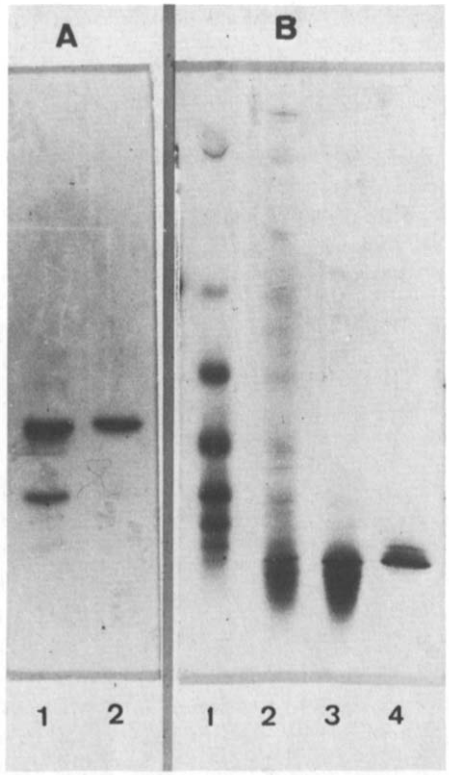

Fig.2. Analytical isoelectric focusing. The pH gradient was 3.5 (bottom) to 9.5. (A) Partially purified CBH II before (lane 1) and after (lane 2) affinity chromatography. (B) Crude cellulase preparation before and after affinity chromatography: 1, cellulobiose-fraction (CBH II isoenzymes); 2, crude cellulase; 3, lactose-fraction (CBH I isoenzymes); 4, CBH I isoenzyme (p*I* 3.9) obtained by ion-exchange chromatography [2].

compounds effectively suppressed the β-glucosidase activities and elution of the adsorbed fractions was then performed successively with 0.1 M lactose and 0.01 M cellobiose. The isoelectric focusing patterns of both eluted fractions are shown in fig.2.

The characteristic isoenzyme pattern of CBH I is observed for the 'lactose-fraction', whereas the 'cellobiose-fraction' shows a series of bands – presumably the CBH II isoenzyme pattern – characteristic of the present cellulase preparation. The specific activities of several fractions using the standard substrates are compared in table 1.

In conclusion, the affinity support described here offers a rapid and efficient method for the group-separation of the two exocellobiohydrolases from crude *T.reesei* culture filtrate by selective adsorption and elution. It seems to be a very favorable alternative to methods such as ion-exchange chromatography and preparative isoelectric focusing, which are far more expensive and time-consuming. Also this method minimizes the risk of enzyme denaturation. The addition of appropriate inhibitors prevents possible column deterioration when crude samples are applied. An efficient one-step procedure for screening culture filtrates from cellulolytic microorganisms is thereby provided.

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Table 1

Activities in fractions eluted from the affinity column

Fraction eluted with	Specific activities (μmol glucose·min^{-1}·mg^{-1})		Ratio
	Avicelase[a]	CMC'ase[b]	
Buffer (unretarded)	0.0059	0.2828	48
Lactose (CBH I)	0.0175	0.0099	0.56
Cellobiose (CBH II)	0.0391	0.0165	0.42

[a] Activities at 50% were determined with a 1% suspension of Avicel in 0.05 M sodium acetate buffer (pH 5.0). One unit of activity was defined as the amount of enzyme to liberate reducing sugars corresponding to 1 μmol glucose per min under these conditions. Specific activities are expressed per mg protein

[b] Activities were similarly assayed with 1% solutions of carboxymethylcellulase (CMC, sodium salt; low-viscosity grade) in 0.05 M sodium buffer, pH 5.0 (50°C)

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